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Expression of histidine-tagged bovine growth hormone employing the prokaryotic pET system

Alaín González Pose[1,2]‡*, Elianet Lorenzo Romero[2]*, Dailenis Abella Matos[2], Mary Karla Méndez Orta[2], Liliana Basabe Tuero[2], Ernesto Manuel González Ramos[2], Anays Álvares Gutiérrez[2], Raquel Montesino [1], Oliberto Sánchez [3], Jorge Roberto Toledo [1].

**Both authors contributed equally to this investigation*
[1]Biotechnology and Biopharmaceutical Laboratory, Pathophysiology Department, School of Biological Sciences, Universidad de Concepción, Victor Lamas 1290, P.O. Box 160-C, Concepción, Chile.
[2]Animal Biotechnology Department, Center for Genetic Engineering and Biotechnology (CIGB), P. O. Box 6162, Havana 10600, Cuba.
[3]Recombinant Biopharmaceuticals Laboratory, Pharmacology Department, School of Biological Sciences, Universidad de Concepción, Victor Lamas 1290, P.O. Box 160-C, Concepción, Chile.
‡Corresponding author: Alaín González Pose

Abstract: *As the recombinant bovine growth hormone (rbGH) has become crucial in galactopoiesis research, the main goal of this study was the in vitro generation of histidine-tagged rbGH proteins using the prokaryotic pET system. The isolation of rbgh genes was carried out by PCR using specific primers and a cDNA template retrotranscribed from a sample of total RNA extracted from the bovine pituitary tissue. The PCR products were cloned into the expression vectors pET28a and pET22b. The genetic construction in pET28a added six-histidine residues at the N-terminus region of the rbGH molecule. The genes inserted in pET22b allowed the obtaining of rbGH protein without histidine residues and another variant with the six-histidine residues at the C-terminus region. Different E. coli strains were transformed with the three genetic constructions. The strain Rosetta™ (DE3) was selected as the final expression system because it exhibited the highest expression levels among others. After purifying the three rbGH variants, densitometric analysis showed more than 95 % purity in all cases. The proteins also showed biological activity in a cell proliferation assay. This study allowed the obtaining of three actives variants of rbGH with a high purity degree by using the prokaryotic pET system and the E. coli strain Rosetta™ (DE3). This approach could contribute to the production of rbGH protein for future galactopoiesis investigations.*

Keywords: *bovine growth hormone; bovine somatotropin; histidine-tag; pET system.*

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I. Introduction

The bovine growth hormone (bGH) or bovine somatotropin is a single-chain polypeptide of 191 amino acids with a molecular mass of 22 kDa secreted by the anterior pituitary gland [1-3]. The bGH encoding gene is located in the 19q26 bovine chromosomal region [4] and it has 1800 base pairs approximately, comprising five exons and four introns [5, 6]. This hormone has several biological functions, such as: normal body growth, tissue development, fat metabolism and reproduction [2, 7, 8]. Also, in 1937 was identified an important role of bGH in lactation [9]. Although the galactopoietic effect of bGH in lactating ruminants is well established [10], the molecular mechanisms responsible for the increasing of milk production are not fully understood [11]. It has been suggested an indirect effect of the hepatic IGF-1 in the synthesis of milk proteins by increasing the mammary blood flow. Also, this growth factor could mobilize nutrients from muscle and adipose tissue to the mammary glands and reduce the amino acid oxidation [10]. Moreover, bGH receptor has been found in mammary epithelial cells [12]. Hence, bovine mammary glands can respond directly to bGH treatment [13]. In the 1980s, it became technically possible to produce large amounts of bGH at low costs using the recombinant DNA technology. Since 1993, the Food and Drug Administration (FDA) authorized the use of recombinant bGH (rbGH) as an alternative treatment to increase dairy production in breastfeeding cows [14]. Although the use of rbGH is controversial [15], it is considered that cattle treated with rbGH has provided nutritious and healthy milk to the population, with consequent economic and environmental benefits [16-18]. The use of rbGH in lactating cattle has allowed the dairy industry to increase the milk production in 10-15 % [19]. On the other hand, it has been demonstrated that rbGH can be biologically active in other species, like: goat, sheep and fish [19]. Lactating sheep increases the milk

production up to 27 % after the rbGH treatment [20]. Also, rbGH can enhance the fish growth rate by 40-60 % [21-23]. Due to applications of rbGH protein in the agricultural sector, some biological research have been directed to obtain this molecule in several expression systems, such as: bacteria [24-26], yeast [27], fungus [28], mammalian cells [29], transgenic plants [30] and transgenic animals [31]. In this study, recombinant bgh (rbgh) genes were cloned into the prokaryotic pET system for expressing histidine-tagged rbGH proteins in different *E. coli* strains. The rbgh genes with six-histidine residues at N-terminus, C-terminus and without histidine residues were expressed in the *E. coli* strains: BL-21 CodonPlus-RIL, BL-21 CodonPlus-RP and Rosetta™ (DE3). The latter strain was selected as the final expression system because it showed the highest expression levels of the three rbGH variants among other hosts. These proteins were scaled up and purified, obtaining more than 95 % purity. Also, their biological activities were tested by a cell proliferation assay.

II. Materials and Methods

Isolation of rbgh genes.

The pituitary tissue was extracted from cows sacrificed in the Nueva Paz slaughterhouse, Mayabeque, Cuba. One hundred milligrams of pituitary tissue were mixed at 6000 rpm for 5 minutes in the homogenizer T25 basic Ika Labortechnik. Total RNA was extracted with TriReagent (Sigma, U.S.A.) following the manufacturer's recommendations. Complementary DNA (cDNA) was obtained from 1 μg of total RNA using the kit Reverse Transcription System (Promega, U.S.A.). The genes coding for rbGH proteins were amplified by PCR using an automatic Master cycler (Eppendorf, U.S.A.) and the Platinum®Pfx DNA polymerase (Invitrogen, U.S.A.). Two PCR amplifications were performed using different sets of primers. The primers: forward (11-563) 5´-GCCTTCCCAGCCATGTCCTTGTCC-3´ and reverse (11-564) 5-CTGGCAAC TAGAAGGCACAGCTGGC-3´, were used to obtain the rbgh gene with six-histidine residues at the N-terminus and without histidine residues. The primers: forward (13-188) 5´-CATATGGCT TTCCCGGCAATGTCCTTGTCC-3´ and reverse (13-189) 5´-GAATTCATAAAGGCACAGCTGG CTTCCCCG-3´ were used to obtain the rbgh gene with six-histidine residues at the C-terminus. The PCR reactions were conducted under the following conditions: five minutes at 94°C, followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 63°C and one minute at 68°C. A final polymerization step of five minutes at 68°C was added.

Cloning and expression of rbgh genes.

The PCR products, previously phosphorylated, were subcloned into the plasmid pMOS-Blue (Sigma, U.S.A.), obtaining the plasmids pMOS-rbgh1 and pMOS-rbgh2. The expression vector carrying the rbgh gene with six histidine residues at the N-terminus (pET28a-rbghN) was obtained by removing the rbgh gene from the plasmid pMOS-rbgh1 with the enzymes *Nde* I (Promega, U.S.A.) and *BamH* I (Promega, U.S.A.) and inserting it into the prokaryotic expression vector pET28a (Invitrogen, U.S.A.), previously digested with the same enzymes. The expression vector carrying the rbgh gene without the six histidine residues (pET22b-rbghA) was obtained by removing the rbgh gene from the plasmid pMOS-rbgh1 with the enzymes *Nde* I and *EcoR* I (Promega, U.S.A.) and inserting it into the prokaryotic expression vector pET22b (Invitrogen, U.S.A.), previously digested with the same enzymes. The expression vector carrying the rbgh gene with six histidine residues at the C-terminus (pET22b-rbghC) was obtained by removing the rbgh gene from the plasmid pMOS-rbgh2 with the enzymes *Nde* I and *EcoR* I and inserting it into the prokaryotic expression vector pET22b, previously digested with the same enzymes. The five plasmids were sequenced (Macrogen, South Korea) and checked by restriction assays to confirm the authenticity of the genes of interest. The *E. coli* strains BL21-CodonPlus® (DE3)-RIL (Stratagene, U.S.A.), BL21-CodonPlus® (DE3)-RP (Stratagene, U.S.A.) and Rosetta™ (DE3) (Novagen, Germany) were transformed with the expression vectors pET28a-rbghN, pET22b-rbghA and pET22b-rbghC following the procedures of the instruction manual BL21-CodonPlus® Competent Cells (Stratagene, U.S.A.), as well as the expression induction of the different genes. Immunoidentification assays were performed with three antibodies: a rabbit polyclonal Anti-Bovine Growth Hormone antibody (ab31496) (Abcam, U.K.), a monoclonal Anti-Bovine Growth Hormone antibody [8.F.23] (ab30538) (Abcam, U.K.) and a 6xHis monoclonal antibody (Albumin Free) (631212) (Clontech, U.S.A.).Figure 1 shows the amino acid sequences of rbGH proteins.

Solubilization and purification of rbGH proteins.

After scaling up the process to a 2 liter fermenter using the best expression system, 25 g of biomass was resuspended in 300 mL of 50 mM Trsi-HCl (Merck, Germany), pH 9 for cell lysis by two rounds of 10 MPa in French press (Ohtake, Japan). The solubilization of rbGH proteins was performed as previously described [24], with some modifications. Briefly, 3.5g from the pellet of cellular lysate were resuspended in 50 mL of the solubilization buffer (50 mM Tris-HCl; 0.1 M dithiothreitol (DTT) (Sigma, U.S.A.); 10 M urea (Merck, Germany), pH 9) and heated at 100°C for 5 minutes. The solution was cooled to room temperature and 50 mM Tris-HCl was added until reaching a concentration of 4 M urea. After spinning at 10000xg for 10 minutes, the

pellet was discarded. The proteins rbGHN and rbGHC were purified by immobilized metal affinity chromatography (IMAC). The solutions containing the solubilized proteins were adjusted to 5 mM imidazole (Merck, Germany), and filtered through 0.45 µm pore size before being applied into a column filled with 10 mL of the matrix Chelating Sepharose ™ Fast Flow (Amersham, Sweden).

This matrix was previously loaded with a divalent metal ion solution of 0.1 M $NiSO_4$ (Merck, Germany) and equilibrated with a buffer containing 50 mM Tris-HCl, 0.1 M DTT, 4 M urea, 5 mM imidazole, pH 9 at a flow rate of 0.2 mL/minute. After performing two washes with three volumes of the previous buffer containing 80 and 120 mM imidazole, the proteins were eluted with the same buffer containing 200 mM imidazole. The purification of the protein rbGHA was performed by ion exchange chromatography (IEC) using the ionic exchangers Q and SP Streamline (GE Healthcare, U.S.A.), which were equilibrated with a buffer containing 10 mM Tris-HCl, 0.1 M NaCl (Merck, Germany), 4 M urea, pH 9 at a flow rate of 0.2 mL/minute. After applying the sample with the protein of interest, each ionic exchanger was washed with a buffer containing 10 mM Tris-HCl; 0.1M NaCl, pH 9 and eluted using a continuous gradient, where pH and ionic strength were modified simultaneously. The gradient was automatically executed using one buffer of low ionic strength and high pH (10 mM Tris-HCl, 10 mM NaCl, pH 9) and another with high ionic strength and low pH (10 mM Tris-HCl, 1 M NaCl, pH 3).

rbGHN

MGSSHHHHHHSSGLVPRGSHMDAFPAMSLSGLFANAVLRAQHLHQLAADTFKEFERTYIPE
GQRYSIQNTQVAFCFSETIPAPTGKNEAQQKSDLELLRISLLLIQSWLGPLQFLSRVFTNSLVF
GTSDRVYEKLKDLEEGILALMRELEDGTPRAGQILKQTYDKFDTNMRSDDALLKNYGLLSCF
RKDLHKTETYLRVMKCRRFGEASCAF

rbGHA

MDAFPAMSLSGLFANAVLRAQHLHQLAADTFKEFERTYIPEGQRYSIQNTQVAFCFSETIPA
PTGKNEAQQKSDLELLRISLLLIQSWLGPLQFLSRVFTNSLVFGTSDRVYEKLKDLEEGILAL
MRELEDGTPRAGQILKQTYDKFDTNMRSDDALLKNYGLLSCFRKDLHKTETYLRVMKCRR
FGEASCAF

rbGHC

MDAFPAMSLSGLFANAVLRAQHLHQLAADTFKEFERTYIPEGQRYSIQNTQVAFCFSETIPA
PTGKNEAQQKSDLELLRISLLLIQSWLGPLQFLSRVFTNSLVFGTSDRVYEKLKDLEEGILAL
MRELEDGTPRAGQILKQTYDKFDTNMRSDDALLKNYGLLSCFRKDLHKTETYLRVMKCRR
FGEASCAFIGSHHHHHH

Figure 1: Amino acid sequences of the three rbGH variants. Dotted line: additional amino acids. Double line: six-histidine residues. Line: bGH sequence. The isolated bGH sequence matches with the NM_180996 sequence from the National Center for Biotechnology Information (NCBI).

Proteins were detected using the purification system Flash (Armens, France) coupled to the software Armens Glider. The pooled target protein peak was concentrated in AMICON Millipore (Bedford, U.S.A.) and dialyzed using a buffer containing 50 mM Tris-HCl, 10 mM NaCl, pH 9. The concentration and purity of the proteins were assessed by the Pierce™ BCA Protein Assay Kit (Thermo Scientific, U.S.A.) linked to densitometry. The last analysis was performed by the screening of SDS-PAGE gels (15%) stained with a Coomassie blue R-250 solution at 0,05% using the software TDI's 1D Manager, version 2.0.

Biological activity assay.

Bovine peripheral blood mononuclear cells (BPBMC) were isolated using Histopaque 1083 (Sigma, U.S.A.) and washed three times with PBS (8 g/L NaCl (Merck, Germany), 0.2 g/L KCl (Merck, Germany), 1.09 g/L Na_2HPO_4 (Merck, Germany), 0.2 g/L KH_2PO_4 (Merck, Germany), pH 7.2). BPBMC were seeded in 96-well plates (Costar, U.S.A.) at 2.8 x 10^5 cells/well using RPMI (Gibco, U.S.A.) supplemented with 10 % fetal bovine serum (PAA Laboratories Inc., Canada) and non-essential amino acids (Sigma, U.S.A.). Cells were treated with the three rbGH variants at 1, 10 and 100 ng/well. A commercial bGH (Active cow Bovine Growth Hormone full-length protein ab123464) (Abcam, U.K.) was used as positive control at the same quantities. Also, concanavalin A (Sigma, U.S.A.) at 1µg/mL was used as proliferation control. The dialysis buffer was used as negative control. Biological activity was assessed by the proliferation kit CellTiter 96® Nonradioactive Cell Proliferation Assay (MTT) (Promega, U.S.A.) following the manufacturer's instructions. Absorbance was measured in a microplate reader Sunrise-basic Tecan Austria (Männedorf, Switzerland) at 570 nm. Four experiments were performed using fourteen experimental groups with two replicates per group.

Statistical analysis.

The statistical analysis was performed using the statistical software GraphPad Prism v.6.0e (GraphPad, San Diego, CA, USA). Differences among the experimental groups of the cell proliferation assay were compared by one-way ANOVA test and the multiple comparison Tukey post-test. Significance was considered for $p<0.05$.

III. Results

Isolation and cloning of rbgh genes.

The isolation of rbgh genes was performed by the extraction of total RNA from bovine pituitary tissue (Figure 2 A and B). After obtaining the cDNA from the reverse transcription reaction using the total RNA as template, two PCR reactions with different set of primers were assessed to amplify rbgh genes. Electrophoresis in agarose gels showed the two PCR products as DNA bands of 600 bp approximately (Figure 2 C and D).

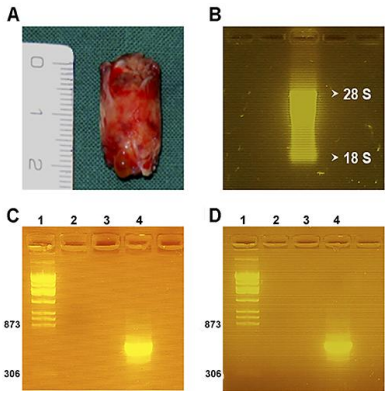

Figure 2: Isolation ofrbgh genes. (A): Bovine pituitary tissue of 2 cm approximately. (B) Total RNA from the pituitary tissue. The subunits 28S and 18S are shown. Electrophoresis in agarose gel (2%) of rbgh genes amplified by PCR with the primers 11-563 and 11-564 (C)or the primers 13-188 and 13-189 (D). 1- Molecular weight marker (plasmid pAdEasy digested with the enzyme *Apa* I). 2- PCR reaction without primers or template. 3- PCR reaction with primers and without template. 4- DNA bands corresponding torbgh genes.

The plasmids pMOS-rbgh1 and pMOS-rbgh2 were obtained by subcloning the PCR bands into the plasmid pMOS-Blue (Figure 3 A). These two plasmids were sequenced and verified by restriction assays using the enzymes *Pvu* II, *Pst* I and *BspH* I (Figure 3 B).

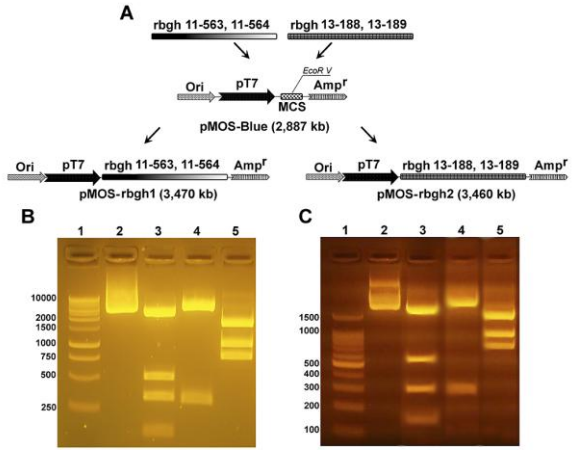

Figure 3: Subcloning ofrbgh genes into the plasmid pMOS-Blue. (A) Representation of the plasmid construction for pMOS-rbgh1 and pMOS-rbgh2. Electrophoresis in agarose gel (2%) of the restriction analysis for plasmids pMOS-rbgh1 (B) and pMOS-rbgh2(C) with different restriction enzymes. 1- Molecular weight marker (1kb DNA Ladder (B) and 100 bp Ladder (C)), 2- Plasmid pMOS-Blue undigested, 3- *Pvu* II digestion, 4- *Pst* I digestion and 5- *BspH* I digestion.

The DNA bands obtained after electrophoresis in agarose gel showed the expected restriction pattern for each plasmid**.** Further cloning were performed in order to obtain the expression vectors using the prokaryotic pET system for expressing rbgh genes. The rbgh gene from the plasmids pMOS-rbgh1 was inserted into the expression vectors pET28a and pET22b, obtaining the plasmids pET28a-rbghN for expressing the rbgh gene with six-histidine residues at the N-terminus and pET22b-rbghA for expressing the rbgh gene alone. The rbgh gene from the plasmids pMOS-rbgh2 was also inserted into the expression vectors pET22b, obtaining the plasmids pET22b-rbghC for expressing the rbgh gene with six-histidine residues at the C-terminus. After corroborating the rbgh genes identity by sequencing, the restriction assay of the three expression vectors with the enzyme *Pvu* II showed the expected band pattern (Figure 4 A, B and C).

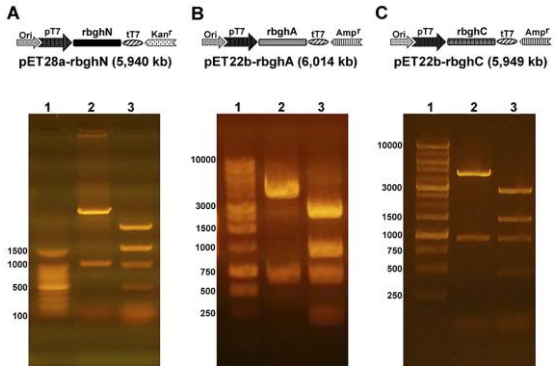

Figure 4: Construction of the expression vectors. Cloning representationofrbgh genes and electrophoresis in agarose gel (1%) of the restriction analysis for plasmids pET28a-rbghN (A), pET22b-rbghA (B) and pET22b-rbghC (C) with the restriction enzyme Pvu II. 1- Molecular weight marker (100 pb Ladder (A) and 1kb DNA Ladder (B, C)), 2-Undigested plasmids (pET28a (A) and pET22b (B, C)), 3- *Pvu* II digestion.

Expression and purification of rbGH proteins.

Once assembled the expression vectors for expressing the three variants of rbgh genes, the *E. coli* strains BL21-CodonPlus® (DE3)-RIL, BL21-CodonPlus® (DE3)-RP and Rosetta™ (DE3) were transformed with the three expression vectors to obtain the recombinant proteins rbGHN, rbGHA and rbGHC. The SDS-PAGE assays showed the expression of the three rbgh genes in the three *E. coli* strains. The proteins rbGHN and rbGHC were observed at 24 kDa and the protein rbGHA was observed at 22 kDa, approximately (Figure 5 A).

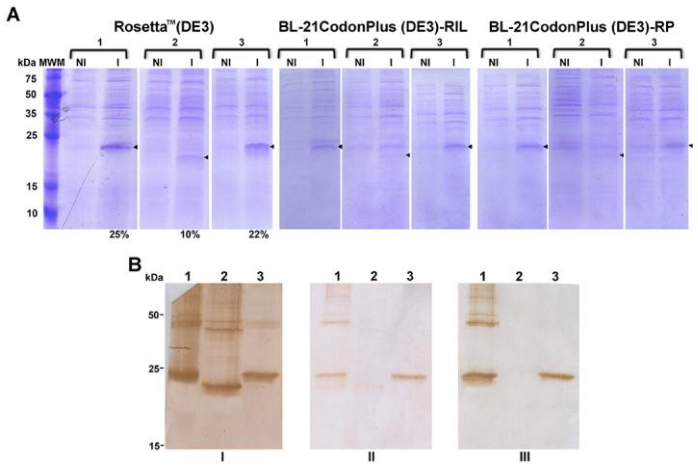

Figure 5: Expression of genes coding the proteins rbGHN, rbGHA and rbGHC in different *E.coli*strains. (A)SDS-PAGE (15%) of the total proteins induced (I) or not (NI) in the *E. coli* strains BL21-CodonPlus® (DE3)-RIL, BL21-CodonPlus® (DE3)-RP and Rosetta™ (DE3) after being transformed with the plasmid pET28a-rbghN(1),pET22b-rbghA (2) and pET22b-rbghC (3). MWM: Molecular weight marker (Promega V8491). (B) Western-blot of the proteins rbGHN (1), rbGHA (2) and rbGHC (3) produced in the Rosetta™ (DE3)strain. I: Polyclonal antibody against bGH, II: Monoclonal antibody against bGH,III: Monoclonal antibody against the six-histidine residues. Arrowheads in SDS-PAGE indicate the different variants of rbGH proteins.

Densitometric analysis revealed the highest expression levels in the *E. coli* strain Rosetta^{TM} (DE3), which expressed the proteins rbGHN, rbGHA and rbGHC at 25 %, 10 % and 22 %, respectively. Therefore, the Rosetta^{TM} (DE3)strain was selected as the final expression system.

The Western-blot assay with three different antibodies demonstrated the authenticity of the proteins rbGHN, rbGHA and rbGHC expressed in the *E. coli* strain Rosetta^{TM} (DE3), showing the presence of dimeric and multimeric forms (Figure 5 B).

After scaling up the process to increase the protein production, cell lysis was performed to extract the three recombinant proteins. SDS-PAGE showed that most of the proteins remained in the cellular pellet (Figure 6 A). A treatment with urea and DTT allowed the solubilization of the proteins (Figure 6 B).

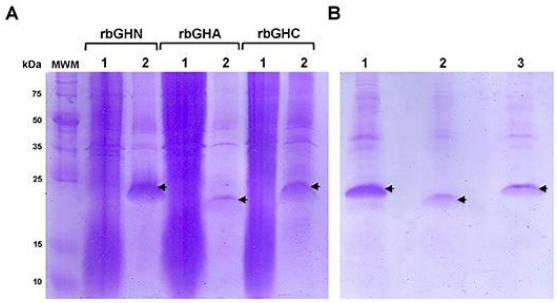

Figure 6: Solubilization of proteins rbGHN, rbGHA and rbGHC. (A)SDS-PAGE (15%) of the supernatant (1) and the pellet (2) after celllysis. MWM: Molecular weight marker (Promega V8491). (B) SDS-PAGE (15%) of soluble proteins rbGHN (1), rbGHA (2) and rbGHC (3) after the treatment of pellet lysatewith urea and DTT. Arrowheads indicate the different variants ofrbGH proteins.

Once solubilized, the three rbGH variants were submitted to purification processes. The proteins rbGHN and rbGHC were purified by IMAC. The protein rbGHA was purified by IEC. Although low amounts of the proteins were lost in the unbound material and in the wash steps (not shown), the SDS-PAGE showed that proteins were obtained with more than 95 % purity according to the densitometric analysis (Figure 7).

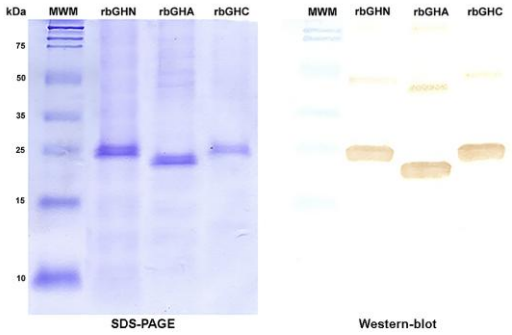

Figure 7: Purification of proteins rbGHN, rbGHA and rbGHC. SDS-PAGE (15 %) and Western-blot of purified proteins. The proteins rbGHN and rbGHC were purified by IMAC and the protein rbGHA was purified by IEC. MWM: Molecular weight marker (Promega V8491). The polyclonal antibody against bGH was used for immunoidentification.

Biological activity of rbGH proteins.

In order to know if the three proteins were biologically active, an assay of cell proliferation using BPBMC was performed. The BPBMC were treated with the proteins rbGHN, rbGHA and rbGHC at 1, 10 and 100 ng. Concanavalin A was used as proliferation control and a commercial bGH was used as positive control. The negative control were the BPBMC treated with the dialysis buffer. The three rbGH variants behave similar to the commercial bGH used as positive control. The biological activity followed a dose-dependent pattern. There were significant differences in cell proliferation when 10 and 100 ng of the rbGHN and rbGHA were compared with the negative control. However, the rbGHC only showed significant differences when the BPBMC were treated with 100 ng of the protein.

IV. Discussion

Milk is considered as a complete nutritional source [17]. One of the approaches for increasing its production is the hormonal treatment of lactating cows with bGH produced by the recombinant DNA technology [16-18]. As one of our goals is to study galactopoiesis in cattle using rbGH, the objective of this research was the *in vitro* generation of three variants of the rbGH protein using the prokaryotic pET system.

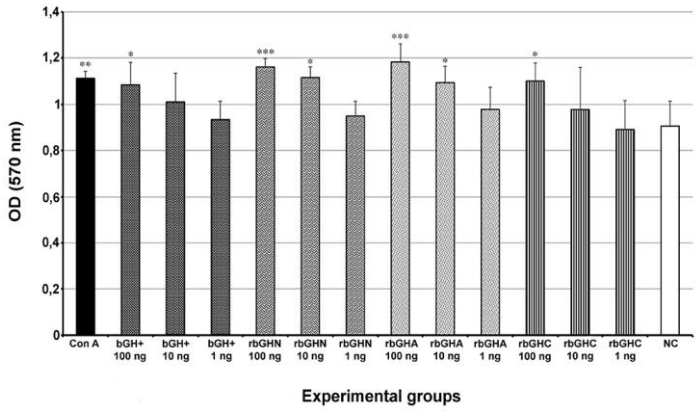

Figure 8: Cell proliferation assay using bovine peripheral blood mononuclear cells (BPBMC). The proteins rbGHN, rbGHA and rbGHC were added at 1, 10 and 100 ng/well. OD values were compared using an ANOVA test and a Tukey post-test. Data represent the arithmetic mean of four experiments with two replicates per group. Bars represent the standard deviation. Con A: Concanavalin A at 1 µg /mL, proliferation control. bGH+: Commercial bGH, positive control. NC: Negative control.* (p<0.05), ** (p<0.01), *** (p<0.001).

Four variants of bGH hormone are naturally released from the pituitary gland, which include phenylalanine (190 amino acids) or alanine (191 amino acids) at the N-terminus due to differential processing and valine or leucine at position 127 caused by allelic polymorphism [32-34]. The amino acid sequence of the bGH protein isolated in this study revealed an alanine at the N-terminus and a leucine at position 127.After isolating rbgh genes, the pET system was selected for their expression because it is one of the most powerful systems for cloning and expressing recombinant proteins in *E. coli*. This system can overproduce a heterologous protein in more than 50 % of the total cell protein [35]. The cloning of rbgh genes in the expression vectors pET28a and pET22b allowed the addition of six-histidine residues at the N- and C- terminus of the molecule.

Currently, the histidine tag is a useful tool for expressing heterologous proteins due to its ability for establishing an antibody mediated detection system for unknown proteins and the possibility of performing an easy purification method by affinity to metals. After obtaining the expressions vectors with the three variants of rbGH (rbGHN, rbGHA and rbGHC), an *E. coli* strain had to be selected as a host. It is known that rare codons from heterologous proteins could impair their expression in *E. coli* strains [36, 37]. As rbgh genes used in this study were isolated from a natural source, an analysis of rare codons for the bgh sequence was performed. A detailed scrutiny revealed eleven rare codons consisting in three distinct variants for arginine, one for glycine and one for proline (Figure 9).

Figure 9: Nucleotide sequence ofthe bgh gene highlightingeleven rare codons. Line: rare arginine codons (CGG, AGA, AGG). Double-line: rare glycine codon (GGA). Dot-line: rare proline codon (CCC).

Taking into account this information, specific *E. coli* strains carrying rare tRNAs (BL21-CodonPlus® (DE3)-RIL, BL21-CodonPlus® (DE3)-RP and Rosetta™ (DE3)) were selected to express the three rbGH variants. The highest expression levels were achieved by the *E. coli* strain Rosetta™ (DE3), probably because it was the only strain containing all the rare codons of rbgh genes isolated. The immunoidentification of the three rbGH variants showed dimeric structures, which could increase the biological activity of the proteins [38]. After solubilizing and purifying rbGH proteins, they were submitted to a biological activity assay. The rbGH has the property of stimulating bovine lymphocyte proliferation [39]. Hence, BPBMC were selected to perform a cell proliferation assay. There were similarities in the action pattern of the three rbGH proteins, showing an increase of cell proliferation in a dose-dependent manner. Although the protein rbGHC showed a little less activity, this experiment demonstrated that the addition of the six-histidine residues at the N- or C-terminus of rbGH proteins did not impair their ability to increase cell proliferation of BPBMC. However, the biological activity of the three rbGH proteins could be different during *in vivo* galactopoiesis assays. The bGH hormone has several domains with distinct functions [40]. The residues from 96 to 133 promote growth, while residues from 1 to 95 and from 134 to 191 have less activity [41]. The N-terminus of human growth hormone (residues from 1 to 15) exerts an insulin-like function [42], while the C-terminus (residues from 177 to 191) induces the opposite effect [43]. As a histidine tag was added at the N- and C-terminus in different rbgh gene constructions, it could modify the *in vivo* effect of these recombinant proteins during galactopoiesis. Additional experiments have to be done with these three rbGH variants to corroborate if they can increase the milk production in lactating cows.

V. Conclusions

Basic investigations about bGH have revealed multiple functions of this hormone. Since its massive production by the recombinant DNA technology was achievable, the rbGH has become an attractive molecule due to its numerous applications in agricultural biotechnology. It is thoroughly known that rbGH exerts a positive effect on galactopoiesis by increasing the milk production upon administration in lactating cows. This approach has been exploited for decades with outstanding results. In this study, three rbGH variantswere obtained using the prokaryotes pET system and the *E. coli* strain Rosetta™ (DE3). All rbGH variants showed a high purity degree after purification and they were biologically active. The combining of histidine tag technology with the prokaryotic pET expression system could provide a fast, easy and cost effective method to produce large amounts of rbGH protein for further galactopoiesis experiments.

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